

1 **SOLID PHASE NATIVE CHEMICAL LIGATION**
2 **OF UNPROTECTED OR N-TERMINAL CYSTEINE PROTECTED PEPTIDES**
3 **IN AQUEOUS SOLUTION**

4
5 **CROSS-REFERENCE TO RELATED APPLICATIONS**

6 This application claims the benefit of U.S. Provisional Application No. 60/049,553, filed
7 June 13, 1997.

8 **INTRODUCTION**

9 **Background**

10 Existing methods for the chemical synthesis of proteins include stepwise solid phase
11 synthesis, and fragment condensation either in solution or on solid phase. The classic stepwise
12 solid phase synthesis of Merrifield involves covalently linking an amino acid corresponding to
13 the carboxy-terminal amino acid of the desired peptide chain to a solid support and extending the
14 polypeptide chain toward the amino end by stepwise coupling of activated amino acid
15 derivatives having activated carboxyl groups. After completion of the assembly of the fully
16 protected solid phase bound peptide chain, the peptide-solid phase covalent attachment is
17 cleaved by suitable chemistry and the protecting groups removed to give the product
18 polypeptide.

19 Some disadvantages of the stepwise solid phase synthesis method include: incomplete
20 reaction at the coupling and deprotection steps in each cycle results in formation of solid-phase
21 bound by products. Similarly, side reactions due to imperfections in the chemistry, and or
22 impurities present in the reagents/protected amino acids, all lead to a multiplicity of solid phase
23 bound products at each step of the chain assembly and to the formation of complex product
24 mixtures in the final product. Thus, the longer the peptide chain, the more challenging it is to
25 obtain high-purity well-defined products. Due to the production of complex mixtures, the
26 stepwise solid phase synthesis approach has size limitations. In general, well-defined
27 polypeptides of 100 amino acid residues or more are not routinely prepared via stepwise solid
28 phase synthesis. Synthesis of proteins and large polypeptides by this route is a time-consuming
29 and laborious task.

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1 The solid phase fragment condensation approach (also known as segment condensation)
2 was designed to overcome the difficulties in obtaining long polypeptides via the solid phase
3 stepwise synthesis method. The segment condensation method involves preparation of several
4 peptide segments by the solid phase stepwise method, followed by cleavage from the solid phase
5 and purification of these maximally protected segments. The protected segments are condensed
6 one-by-one to the first segment, which is bound to the solid phase.

7 Often, technical difficulties are encountered in many of the steps of solid phase segment
8 condensation. See E. Atherton, et al., "Solid Phase Fragment Condensation - The Problems," in
9 Innovation and Perspectives in Solid Phase Synthesis 11-25 (R. Epton, et al. 1990). For
10 example, the use of protecting groups on segments to block undesired ligating reactions can
11 frequently render the protected segments sparingly soluble, interfering in efficient activation of
12 the carboxyl group. Limited solubility of protected segments also can interfere with purification
13 of protected segments. See K. Akaji et al., Chem. Pharm. Bull.(Tokyo) 33:184-102 (1985).
14 Protected segments are difficult to characterize with respect to purity, covalent structure, and are
15 not amenable to high resolution analytical ESMS (electrospray mass spectrometry) (based on
16 charge). Racemization of the C-terminal residue of each activated peptide segment is also a
17 problem, except if ligating is performed at Glycine residues. Moreover, cleavage of the fully
18 assembled, solid-phase bound polypeptide from the solid phase and removal of the protecting
19 groups frequently can require harsh chemical procedures and long reaction times that result in
20 degradation of the fully assembled polypeptide.

21 Segment condensation can be done in solution rather than on solid phase. See H.
22 Muramatsu et al., Biochem. and Biophys. Res. Commn. 203(2):1131-1139 (1994). However,
23 segment condensation in solution requires purification of segments prior to ligation as well as
24 use of protecting groups on a range of different side chain functional groups to prevent multiple
25 undesired side reactions. Moreover, the ligation in solution does not permit easy purification and
26 wash steps afforded by solid phase ligations. Furthermore, the limitations with respect to
27 solubility of protected peptide segments and protected peptide intermediate reaction products are
28 exacerbated.

29 Chemical ligating of minimally protected peptide segments has been explored in order to
30 overcome the solubility problems frequently encountered with maximally protected peptide

1 segments. See Cheng, et al., Chemical Synthesis of Human δ -endorphin(1-27) Analogs by
2 Peptide Segment Coupling. *Int. J. Pept. Protein Res.* 38:70-78 (1991); J. Blake, Total Synthesis
3 of S-Carbamoylmethyl Bovine Apocytochrome c by Segment Coupling, *Int. J. Pept. Protein Res.*
4 27:191-200 (1986); and H. Hojo et al., Protein Synthesis using S-Alkyl Thioester of Partially
5 Protected Peptide Segments, Synthesis of DNA-Binding Protein of *Bacillus stearothermophilus*,
6 *Bull. Chem. Soc. Jpn.* 65:3055-3063 (1992). However, this method still requires the use of
7 protecting groups on all Lysine side chain amino groups, selective N- α protection of one or more
8 segments, and laborious purification steps, involving purification, reprotection, and
9 repurification.

10 The use of multiply protected peptide segments is incompatible with the overall scheme
11 of engineering proteins using peptides produced by means of recombinant DNA expression as a
12 source. Protected peptide segment methods are labor-intensive, and the protected peptide
13 segments have unpredictable handling properties, partly due to the solubility and ligating
14 difficulties of protected peptide segments. Often, large protected peptide segments are
15 minimally soluble in even the most powerful polar aprotic solvents such as dimethylsulfoxide
16 (DMSO) and dimethylformamide (DMF). The problem of insolubility in protected peptide
17 segments has been addressed with limited success in several ways, including the use of (1)
18 partial protecting group strategy which masks all side chains except those of Ser, Thr, and Tyr;
19 (2) minimal protecting group strategy that masks only thiol and amino side chains; and (3) using
20 reversible protection of a backbone amide moiety to prevent aggregation/insolubility. Protecting
21 groups used in the latter approach alter peptide conformations. Use of backbone protecting
22 groups is not yet straightforward or predictable and requires significant experimentation for each
23 target polypeptide chain.

24 There are a number of techniques for ligating unprotected peptide segments via unnatural
25 backbone linkages. In contrast, there are few methods for achieving a "native chemical
26 ligation." A "native chemical ligation" is the chemoselective reaction of unprotected or N-
27 terminal Cysteine protected peptide segments with another unprotected peptide segment
28 resulting in the formation of a ligated peptide with an amide bond at the ligation site. The fully
29 assembled target polypeptides of the invention comprise one, two or more native chemical
30 ligation sites.

1 Accordingly, there is a need in the art for rapid methods of synthesizing assembled
2 polypeptides via chemical ligation of two or more unprotected peptide segments using a solid
3 support, with improved yields and facilitated handling of intermediate products.

4 The present invention makes possible, inter alia, the rapid solid-phase synthesis of large
5 polypeptides with a natural peptide backbone via native chemical ligation of two or more
6 unprotected peptide segments where none of the reactive functionalities on the peptide segments
7 need to be temporarily masked by a protecting group. The present invention accomplishes for
8 the first time, solid phase sequential chemical ligation of peptide segments in an N-terminus to
9 C-terminus direction, with the first solid phase-bound unprotected peptide segment bearing a C-
10 terminal α -thioester that reacts with another unprotected peptide segment containing an N-
11 terminal Cysteine and a C-terminal thioacid.

12 Other embodiments of the invention also permit solid-phase native chemical ligation in
13 the C- to N-terminus direction, with temporary protection of N-terminal cysteine residues on an
14 incoming (second) peptide segment. Those of ordinary skill in the art will readily appreciate that
15 the invention may also include the use of nonnative chemical ligation to sequentially ligate
16 peptide segments via unnatural linkages on a solid phase. Alternatively, the invention may
17 include the use of native chemical ligation of peptide segments wherein said peptide segments
18 comprise one or more unnatural backbone linkages.

19

20 References

21 Matthys J. Janssen, "Thiolo, Thiono, and Dithio Acids and Esters," Chptr. 15 of The
22 Chemistry of Carboxylic Acids and Their Esters (1969).

23 Schnolzer et al., Science 256:221-225 (1992)

24 Rose et al. J. Am Chem. Soc. 116:30-34 (1994)

25 Liu et al., Proc. Natl. Acad. Sci. USA 91:6584-6588 (1994).

26 Dawson et al. Science 266:77-779 (1994).

27 PCT/US95/05668, WO 96/34878

28 Sakakibara S., Biopolymers (Peptide Science), 37:17-28 (1995).

29 Tam et al., PNAS USA, 92:12485-12489 (1995).

SUMMARY OF THE INVENTION

1
2 The present invention provides, inter alia, novel methods of producing large polypeptides
3 by native chemical ligation of peptide segments in aqueous solution to an unprotected solid
4 phase bound peptide without need for protecting groups on the peptide segments, or, with
5 temporary protection of the N-terminal cysteine of incoming peptide segments. Among the
6 many advantages of this embodiment of the invention are: ease of purification of the
7 intermediate and final products; faster ligation reactions; rapid synthesis of large polypeptides
8 with a natural peptide backbone; ease of ligating reactions due to the lack of protecting groups
9 and resultant enhanced solubility of peptide segments in aqueous or mixed aqueous/organic
10 solutions; chemoselective ligation due to the lack of reactivity of the thioester moiety with other
11 functional groups present in both reactive peptide segments to form stable co-products, resulting
12 in a purer final product without side reactions; adaptability to monitoring on the solid phase via
13 MALDI mass spectrometry or ESI MS (electrospray ionization mass spectrometry); decreased
14 racemization due to the use of mild activation using a thioester and the avoidance of elevated
15 pHs; the polypeptide product is obtained directly in unprotected form; and adaptability to
16 automation and combinatorial techniques.

17 A significant advantage of the solid phase ligations over solution ligations is that the solid
18 phase ligation methods do not require arduous HPLC (high pressure liquid chromatography)
19 purification and lyophilization steps after each ligating reaction, whereas ligations in solution do.
20 Thus, the solid phase ligations eliminate many time-consuming purification steps that decrease
21 the recovery of final product. Instead, the solid phase sequential ligation methods here described
22 only require a single HPLC purification and lyophilization step after the final unprotected
23 peptide segment has been ligated and the assembled peptide is cleaved from the solid phase. The
24 elimination of these time-consuming purification steps allows for faster synthesis of the final
25 product, i.e. the assembled peptide, than would the analogous route in solution. Ready
26 purification of the desired solid phase-bound product from soluble coproducts presents a
27 tremendous advance in terms of the yield of the ultimate assembled polypeptide.

28 Another advantage of solid phase ligations is that they permit higher concentrations of
29 reactants which leads to faster reaction rates. For example, by using an excess at high
30 concentration of the incoming peptide segment as compared to the solid phase-bound peptide,

1 reactions can reach completion much faster. The excess peptide segment can readily be washed
2 off the solid phase after the ligation reaction is complete. Increased yields of final product can
3 be accomplished by increasing concentrations of peptide segments. For example, the solid
4 phase-bound polypeptide can be dried out on the solid-phase and ressolvated in ligation solution.
5 Alternatively, the solid phase-bound peptide can be washed with a solution of incoming peptide
6 segments at high concentration.

7 Other advantages of the present invention are that it allows for synthesis of much larger
8 peptides and proteins than are presently attainable by conventional methods, it is amenable to
9 automation, and the use of high resin loadings allow for easy scale up. Moreover, ligation in the
10 N- to C-terminal direction permits the use of crude peptide segments without need for
11 purification or lyophilization, since termination products formed during stepwise solid phase
12 synthesis of the peptide segments will be unreactive with the solid phase-bound peptide.

13 In one embodiment, the invention comprises a method of producing an assembled
14 peptide having a native peptide backbone by ligating peptide segments in the N- to C-terminal
15 direction, comprising: a) covalently binding an unprotected first peptide segment to a solid
16 phase via a linker comprising a cleavable moiety, wherein said cleavable moiety is stable under
17 ligation conditions and said unprotected first peptide segment is bound to said cleavable moiety
18 at its N-terminus and has an α -thioester at its C-terminus; b) optionally introducing a second
19 unprotected peptide segment, wherein said second segment comprises a cysteine residue at its N-
20 terminus and a thioacid at its C-terminus, under conditions suitable to permit ligation between
21 said first unprotected peptide segment and said second unprotected peptide segment to form a
22 natively ligated peptide bound to said solid phase, wherein said solid phase-bound peptide
23 comprises a thioacid at its C-terminus, and subsequently converting said solid phase-bound
24 peptide thioacid to a thioester; (c) optionally repeating step (b) with additional unprotected
25 peptide segments; (d) introducing a final unprotected peptide segment, comprising a cysteine
26 residue at its N-terminus, under conditions suitable to permit ligation between said solid phase-
27 bound peptide and said final unprotected peptide segment. In a preferred embodiment, the
28 cleavable moiety is cleaved to release the solid phase-bound peptide in the form of the assembled
29 peptide. In another preferred embodiment, cleavable moiety is a cleavable linker capable of
30 being cleaved for purposes of monitoring the sequential ligation reactions. In another

1 embodiment, the first unprotected peptide segment is added as a peptide- α COSH thioacid and
2 subsequently converted to a thioester.

3 The sequential ligation in the N- to C-terminus direction is a surprisingly effective and
4 elegant means of obtaining chemoselective ligation of unprotected peptide segments without
5 racemization. Before the present invention, sequential ligations were not conducted in the N- to
6 C-terminal direction due to concerns regarding racemization at the α COX at the C-terminus of
7 the peptide (peptide- α COX). Using the present invention, the α COSH at the C-terminus of the
8 peptide segment is mildly activated to a thioester and the ligating reaction is carried out in the
9 absence of base, in an aqueous buffered solution, resulting in mild conditions that do not
10 generate racemic mixtures.

11 The methods of the invention can be used for native chemical ligation of peptide
12 segments produced by stepwise solid phase synthesis. The last peptide segment to be added at
13 the C-terminal end of the last solid phase-bound peptide in the reaction scheme may be a
14 recombinantly expressed peptide having an N-terminal Cysteine residue (Cys-recombinant
15 peptide). The thioacid moiety, which is activated to a thioester moiety, can be placed anywhere
-16 a native chemical ligation is desired, including on a side chain. Thus, the sequential ligations of
17 the invention are not limited to linearly assembled peptides.

18 In another embodiment, there is the use of unprotected peptide segment middle pieces
19 each having an N-terminal cysteine residue that participate in native chemical ligation.

20 In another embodiment, the invention comprises a method of producing an assembled
21 peptide having a native peptide backbone by ligating peptide segments in the C- to N-terminal
22 direction, comprising: a) covalently binding an unprotected first peptide segment to a solid
23 phase via a cleavable handle comprising a cleavable moiety, wherein said cleavable moiety is
24 stable under ligation conditions and said unprotected first peptide segment is bound to said
25 cleavable moiety at its C-terminus and has a Cysteine at its N-terminus; b) introducing a second
26 peptide segment, wherein said second segment comprises a cysteine residue at its N-terminus
27 and an alpha-thioester at its C-terminus, and wherein said second peptide segment has a
28 protecting group bound to its N-terminal cysteine residue, under conditions suitable to permit
29 ligation between said first peptide segment and said second N-terminally protected peptide
30 segment to form a natively ligated peptide bound to said solid phase, wherein said solid phase-

1 bound peptide comprises a protecting group bound to an N-terminal cysteine; c) removing said
2 protecting group from solid phase-bound peptide; (d) optionally repeating steps b) and c) with
3 additional peptide segments comprising an N-terminal Cysteine and a C-terminal alpha thioester,
4 wherein said additional peptide segments have a protecting group bound to their N-terminal
5 cysteine residue (e) introducing a final peptide segment, comprising an alpha-thioester at its C-
6 terminus, providing that if said final peptide segment comprises an N-terminal Cysteine, said N-
7 terminal Cysteine is protected by a protecting group, wherein said introducing occurs under
8 conditions suitable to permit ligation between said solid phase-bound peptide and said final
9 peptide segment; and (e) optionally removing said protecting group from the N-terminal cysteine
10 of said solid phase-bound peptide.

11 In another embodiment, the invention comprises a method of producing an assembled
12 peptide having a native peptide backbone by ligating peptide segments in the C- to N-terminal
13 direction, comprising: a) covalently binding an unprotected first peptide segment to a solid
14 phase via a cleavable handle comprising a cleavable moiety, wherein said cleavable moiety is
15 stable under ligation conditions and said unprotected first peptide segment is bound to said
16 cleavable moiety at its C-terminus and has a Cysteine at its N-terminus; b) optionally introducing
17 a second peptide segment, wherein said second segment comprises a cysteine residue at its N-
18 terminus and an alpha-thioester at its C-terminus, and wherein said second peptide segment has a
19 protecting group bound to its N-terminal cysteine residue, under conditions suitable to permit
20 ligation between said first peptide segment and said second N-terminally protected peptide
21 segment to form a natively ligated peptide bound to said solid phase, wherein said solid phase-
22 bound peptide comprises a protecting group bound to an N-terminal cysteine, and subsequently
23 removing said protecting group from solid phase-bound peptide; (c) optionally repeating step (b)
24 with additional peptide segments comprising an N-terminal Cysteine and a C-terminal alpha
25 thioester, wherein said additional peptide segments have a protecting group bound to their N-
26 terminal cysteine residue; (d) introducing a final peptide segment, comprising an alpha-thioester
27 at its C-terminus, providing that if said final peptide segment comprises an N-terminal Cysteine,
28 said N-terminal Cysteine is protected by a protecting group, wherein said introducing occurs
29 under conditions suitable to permit ligation between said solid phase-bound peptide and said

1 final peptide segment; and (e) optionally removing said protecting group from the N-terminal
2 cysteine of said solid phase-bound peptide.

3 In yet another embodiment, there is the solid phase sequential ligation of peptide
4 segments in either or both directions, using a cleavable linker to monitor the ligation reactions
5 via mass spectrometry and to purify the assembled peptide from the solid phase.

6 Another embodiment is a method of bidirectional solid phase native chemical ligation,
7 comprising providing a first peptide segment bound to a solid support via one of its internal
8 amino acid residues, wherein said first peptide segment comprises an N-terminal Cysteine and a
9 C-terminal thioester, and ligating a second peptide segment to either terminus.

10 In another embodiment, there is provided a kit comprising an unprotected peptide
11 segment, covalently bound via an internal amino acid side chain functional group to a cleavable
12 handle, wherein said cleavable handle is linked to a solid phase via a chemoselective functional
13 group complementary to a chemoselective functional group on the solid phase. Said kit can be
14 used for solid phase chemical ligation of unprotected or N-terminal cysteine-protected peptide
15 segments to the solid phase-bound peptide. A preferred example of such a cleavable handle is
16 a functionalized cleavable handle, X-aminoethylsulfonyl ethoxycarbonyl (wherein
17 $X = \text{CH}_3\text{COCH}_2\text{CH}_2\text{CH}_2\text{CONHCH}_2\text{-MSC-}$ or $X = \text{AOA-NHCH}_2\text{-MSC-}$. (AOA =
18 aminooxyacetal).

19 In another embodiment, there are methods of using bromoacetic acid or iodoacetic acid to
20 convert a peptide segment thioacid (peptide- αCOSH) to a thioester (peptide- αCOSR), on a solid
21 phase.

22 In yet another embodiment, there is provided a method of monitoring the solid phase
23 sequential ligation process on the solid phase via MALDI or ESI mass spectrometry, using
24 cleavable linkers. Monitoring via ESI MS can also be accomplished using a TFA-cleavable
25 linker or, when MALDI is the mass spectrometric method used, a photocleavable linker may
26 preferably be used.

27 In a further embodiment, there are provided novel methods of preparing modular large
28 peptide or protein libraries using combinations of the aspects of the invention described herein.
29 Particularly useful are the methods of solid phase sequential ligation of peptide segments to
30 rapidly synthesize multiple analogs of known proteins or polypeptides.

1 Kits and apparatus for assembling polypeptides and polypeptide libraries by the processes
2 described herein are also provided.

3 One of skill in the art will readily appreciate that each of the embodiments of the
4 invention can be combined with other embodiments to obtain a wide range of useful inventions.
5

6 BRIEF DESCRIPTION OF THE DRAWINGS

7 **FIG. 1** is a schematic diagram of a solid phase native chemical ligation scheme, in the N-
8 to C-terminus direction. In one embodiment, the linker is an MSC handle, which is cleavable yet
9 stable under ligation conditions. In another embodiment, the unprotected first peptide segment is
10 covalently bound to a solid phase (resin) via an aminooxy-ketone linkage.

11 **FIG. 2A** illustrates the stability of a 13-residue peptide- α -COSH with a Cysteine residue
12 at the N-terminus under ligation conditions. The HPLC chromatogram shows that only a small
13 percentage of the peptide cyclized or formed larger aggregates, even after overnight storage
14 under ligation conditions.

15 **FIG. 2B** illustrates the stability of the same 13-residue peptide- α COSH in the presence
16 of a thioester peptide having a molecular weight of 1230.2. The HPLC chromatogram shows
17 that the Cys- α -COSH peptide is adequately stable to use in ligation without significant reaction
18 with itself. Furthermore, such byproducts as are formed in small proportion by reaction of the
19 13-residue peptide- α COSH (having an N-terminal cysteine) with itself are unreactive with a
20 resin-bound peptide α -COSR and are readily removed by simple filtration and washing.

21 **FIG. 3A, 3B and 3C** show HPLC chromatograms of the effect of hydrazine on the
22 removal of the MSC handle from a peptide having an N-terminal Cysteine residue. The peak
23 correlating with the mass of 1708.2 represents the desired peptide with the MSC handle
24 removed. The peak corresponding to the mass of 1814.5 represents a reactive side product
25 formed upon cleavage that can react with the desired peptide without the MSC handle. **FIG. 3A**
26 shows a fairly large peak at the 1814.5 mw when an aliquot of the peptide was placed in 6M
27 guanidine• HCl, 0.1 M NaPi, pH 7.5, then diluted into 1 N NaOH for 2 min., then quenched with
28 1 N HCl. **FIG. 3B** is an HPLC chromatogram of the resulting product when the conditions of
29 **FIG. 3A** are repeated with the inclusion of 50 mM hydrazine in the 6 M guanidine• HCl solution.
30 **FIG. 3C** is an HPLC chromatogram of the resulting product when the conditions of **FIG. 3A** are

1 repeated with 200 mM hydrazine in the 6 M guanidine• HCl solution. Hydrazine scavenges the
2 side product, resulting in a purer product.

3 **FIG. 4** is an HPLC chromatogram of the removal of a cleavable MSC handle from a
4 peptide that does not have an N-terminal Cysteine residue, but rather an N-terminal Leucine
5 residue and a Cysteine residue in its approximate center. The molecular weight of the peptide
6 with the MSC handle is 4022.4 and without the MSC handle, 3745.1. An aliquot of the peptide
7 in 6M guanidine• HCl, 0.1M NaAc, pH 4.6 was diluted into 6M guanidine• HCl, 0.1M NaAc,
8 pH 14 for 2 min., quenched with 6M guanidine• HCl, 0.1M NaAc, pH 2.0. The HPLC shows
9 that an internal reaction with the side product still occurs, to form the peak having a mw of
10 3979.7 (corresponding to the modification by the LEV-NHCH₂- handle), but that the extent of
11 the reaction is less than that occurring with a peptide having an N-terminal Cysteine.

12 **FIG. 5A** is a reaction scheme showing the preparation of the PEGA resin used as the
13 solid support in N- to C-terminal sequential ligations. Steps A and B1 are optional steps to
14 produce a photolabile linker for use with MALDI analysis of the resin samples.

15 **FIG. 5B** is a diagram illustrating a generalized scheme for preparing a solid phase (resin)
16 for use in the solid phase sequential ligations of the invention. Structure 1 is a cleavable linker
17 useful for monitoring the progress of coupling and ligation reactions by mass spectrometry. For
18 example, a photo-cleavable linker can be used for on-resin monitoring by MALDI MS, whereas
19 a TFA cleavable linker can be used for monitoring by electrospray MS. Once structure 1 is
20 coupled to the resin, the protecting group (PG) is removed and a functional moiety (structure 3)
21 capable of chemoselective reaction with the first peptide segment, is added to the resin. Once 3
22 is coupled to the resin, the protecting group is removed to give structure 4, which is ready for
23 chemoselective reaction with structure 5, a peptide modified with a cleavable handle and a
24 functional group capable of reaction with the now modified resin (4). Once all subsequent
25 ligations are complete, the "cleavable handle" is cleaved to release the full length peptide
26 (assembled peptide) from the solid phase.

27 **FIG. 6** is a reaction scheme illustrating the derivatization of Peptide Segment 1 (the N-
28 terminal peptide segment).

29 **FIG. 7A and 7B** are HPLC chromatograms of the coupling of a first unprotected peptide
30 segment (1) to the solid support, in this example, an AOA-functionalized resin (PEGA). FIG.

1 7A is an HPLC of the peptide solution as added to the resin. FIG 7B is an HPLC of the
2 supernatant after reaction of the peptide with a molar excess of the resin overnight. A significant
3 amount of the peptide has been removed from the supernatant, indicating that it has been bound
4 to the resin after the overnight reaction.

5 **FIG. 8A and 8B** are HPLC chromatograms of the same experiments reflected in FIG.
6 7A and 7B, except with Isco resin beads as the solid phase.

7 **FIG. 9A, 9B, and 9C** are analyses of the products after step 1 of this figure, binding of
8 the first unprotected peptide segment to the solid phase. **FIG. 9A** is an analytical HPLC
9 chromatogram of the (base plus hydrazine) cleavage of the resin-bound peptide. **FIG. 9B** is a
10 MALDI mass spectrum of the resin, showing a peak corresponding to (1), the resin-bound
11 peptide. **FIG. 9C** is a MALDI mass spectrum after base cleavage of the linker, showing the lack
12 of a peak corresponding to (1), and showing that no peptide is sticking to the solid phase (resin).

13 **FIG. 10A, 10B, and 10C** are analyses of the products after step 3 of this figure, i.e.,
14 ligating of the second unprotected peptide segment (2) to the resin-bound peptide (1). **FIG. 10A**
15 is an analytical HPLC of the product, resin-bound peptide intermediate, showing a large peak
16 with mass of (1) + (2). **FIG. 10B** is a MALDI mass spectrum of the resin before cleavage of the
17 linker, and **FIG. 10C** is a MALDI mass spectrum of the resin after base cleavage of the linker.

18 **FIG. 11** is an HPLC chromatograph of the desalted, lyophilized peptide product (1+2+3
19 of Table 1) after 2 sequential ligations on a solid phase (Isco resin) in the N- to C-terminal
20 direction. The tallest peak corresponds to the crude, lyophilized product, indicating
21 approximately 36% yield.

22 **FIG. 12A and 12B** are ESI MS (electrospray ionization mass spectra) of the main peak
23 corresponding to the assembled peptide (1+2+3 of Table 1). **FIG. 12B** is a reconstructed display
24 of the mass spectrum of FIG. 12A, showing the mass of the product ligated peptide.

25 **FIG. 13** is an HPLC chromatogram of the desalted, lyophilized peptide (1+2+3) after
26 base cleavage of the linker to remove the assembled peptide from the solid phase (PEGA resin).

27 **FIG. 14A and 14B** are electrospray ionization mass spectra of the 7434 mass peak,
28 wherein **FIG. 14B** is a reconstruction of the mass spectrum of **FIG. 14A**.

29 **FIG. 15A, 15B and 15C** are 3 HPLC chromatograms illustrating that the solid support
30 technique can be used for both purification and ligation. **Figs. 15A and 15B** show solution

1 processing of a crude peptide before and after removal of DNP groups, respectively. Both
2 HPLCs show a crude mixture of peptides. **Fig. 15C** is an HPLC chromatogram of the same
3 peptide solution shown in **FIG. 15A**, after coupling to a solid support, removal of DNP groups
4 and base cleavage from the solid phase, resulting in a significantly purer assembled peptide
5 product.

6 **FIG. 16A and 16B** illustrate the reaction scheme for synthesis of MIF(1-115) via solid
7 phase sequential native ligations in the N-terminal to C-terminal direction.

8 **FIG. 17A** is a reaction scheme for the modification of the N-terminal peptide segment.
9 **FIG. 17B** is a diagram illustrating the modification of the aqueous-compatible solid phase in
10 preparation for coupling the first unprotected peptide segment.

11 **FIG. 18A** is a reaction scheme for the coupling of N-terminal modified MIF(1-59) to a
12 solid phase. **FIG. 18B** is an HPLC chromatogram of the released peptide after base cleavage,
13 having an expected mass of 6271 Da. **FIG. 18C and 18D** are electrospray mass spectra of the
14 main component of the released peptide after cleavage of the cleavable handle. **FIG. 18D** is a
15 reconstruction of **FIG. 18C**.

16 **FIG. 19A** is a diagram of the ligation step to form resin-bound MIF(1-80). **FIG. 19B** is
17 an HPLC chromatogram of the products after cleavage of the cleavable handle. **FIG. 19C and**
18 **19D** are mass spectra of the main components of the released peptide after base cleavage, having
19 an expected mass of 8502 Da. **FIG. 19D** is a reconstructed display of the mass spectrum of **FIG.**
20 **19C**.

21 **FIG. 20A** is a diagram of the ligation step to form resin-bound MIF(1-115). **FIG. 20B** is
22 an HPLC chromatogram of the products after cleavage of the cleavable handle. **FIG. 20C and**
23 **FIG 20D** are mass spectra of the released products after base cleavage, having an expected mass
24 of 12450 Da.

25 **FIG. 21** is a schematic diagram of solid phase ligations in the C- to N-terminus direction.
26 The "resin" represents a solid phase. The triangle and its sideways M-shaped partner are
27 complementary functional groups that chemoselectively form a covalent bond. The "handle" is a
28 cleavable handle that can be cleaved to remove the assembled peptide product from the solid
29 phase. The undulating lines comprise amino acid residues of peptide segments. The "PG"
30 represents a protecting group, which can be placed either on a side chain thiol or on the α -amino

1 group of the N-terminal cysteine. Steps 2 and 3 can be repeated, as indicated by the arrow
2 marked 4, for additional peptide segments. Also, a cleavable linker for purposes of monitoring
3 the coupling and ligating reactions can be added between the "handle" and the "resin."

4 **FIG. 22** is a reaction scheme for solid phase sequential ligation in the C- to N-terminal
5 direction of PLA2G5.

6 **FIG. 23** is a reaction scheme for synthesizing a Cam ester derivative for solid phase
7 sequential ligation in the C- to N-terminal direction.

8 **FIG. 24** is a reaction scheme for synthesizing the C-terminal peptide segment for solid
9 phase sequential ligation in the C- to N-terminal direction.

10 **FIG. 25A, B, and C** is a diagram of a scheme for synthesizing an assembled polypeptide
11 via bidirectional solid phase sequential ligation of two or more peptide segments.

12
13 **FIG 26 A and B** are HPLC chromatographs following the solid phase solid phase native
14 chemical ligation of 3 peptide segments in the N- to C- terminal direction, resulting in the
15 assembled peptide, C5a 1-74.

16 **FIG. 27** is a reaction scheme for synthesis of a C-terminal peptide segment for use in the
17 solid phase native chemical ligations described herein, using a CAM ester cleavable handle to
18 remove the synthesized peptide segment from the solid phase.

19 **FIG. 28A and B** are HPLC chromatographs and reconstructed ESI MS of the assembled
20 peptide resulting from solid phase sequential ligation of 3 peptide segments: peptide segment 1
21 (SEQ ID NO: 2)(CADRKNILA), peptide segment 2 (SEQ ID NO: 3)(CYGRLEEKKG) and
22 peptide segment 3 (SEQ ID NO: 4)(ALTKYGFYG) on solid phase in the C- to N-terminal
23 direction, using Fmoc protecting groups.

24 **FIG. 29A and B** are an HPLC chromatograph and ESI MS, respectively, of the final
25 ligation product, i.e. the first ligation product ligated to the third peptide segment
26 (ALTKYGFYG), resulting from solid phase sequential ligation of 3 peptide segments in the C-
27 to N-terminal direction, using ACM as the protecting group.

28 **FIG. 30A-H** are HPLC chromatographs and reconstructed ESI MS of the steps of
29 synthesizing Phospholipase A2 Group 5, a 118 residue protein, using solid phase sequential
30 native chemical ligation of four peptide segments in the C- to N-terminal direction. The first
31 peptide segment is PLA2G5 88-118; the second is PLA2G5 59-87, the third is PLA2G5 26-58,

1 and the fourth is PLA2G5 1-25. FIG 32A and B are an HPLC chromatograph and reconstructed
2 ESI MS of the first peptide segment, respectively. FIG 32C and D are an HPLC chromatograph
3 and reconstructed ESI MS, respectively, of the ligation product of the first and second peptide
4 segments (PLA2G5 59-118) . FIG 32E and F are an an HPLC chromatograph and
5 reconstructed ESI MS, respectively, of PLA2G5 26-118, the ligation product of PLA2G5 59-118
6 and PLA2G5 26-58 (the third peptide segment). FIG 32G and H are HPLC chromatograph and
7 reconstructed ESI MS, respectively, of PLA2G5 1-118, the assembled polypeptide.

8 9 DESCRIPTION OF SPECIFIC EMBODIMENTS

10 Terminology

11 Amino acids: Amino acids include the 20 genetically coded amino acids, rare or unusual
12 amino acids that are found in nature, and any of the non-naturally occurring and modified amino
13 acids.

14 Aqueous solution: solutions containing water, including up to 8M urea in water, up to
15 6M guanidine• HCl in water, up to 60% acetonitrile in water.

16 Assembled Peptide: the final product of a solid phase sequential or bidirectional ligation,
17 after cleavage of the cleavable handle. The assembled peptide comprises at least two separate
18 peptide segments sequentially ligated on a solid phase. The assembled peptide may or may not
19 have biological activity.

20 Cleavable Handle: A cleavable moiety that is capable of being selectively cleaved to
21 release the assembled peptide from the solid phase. The cleavable handle must be capable of
22 resisting cleavage under conditions suitable for coupling, activating, deprotecting, ligating,
23 washing, and other steps involved in the formation of an assembled peptide. The cleavable
24 handle must also be stable to conditions used to produce the first peptide segment that is capable
25 of being bound to a solid phase, including, for example, stepwise solid phase peptide synthesis.
26 The cleavable handle preferably is located directly adjacent to the first peptide segment such that
27 upon cleavage of the cleavable handle, the desired assembled peptide is released from the solid
28 phase. The cleavable handle may be selected from any of the variety of cleavable handles used
29 by those in the field. See, e.g., L. Canne et al., Tetrahedron Letters, 38(19):3361-3364 (1997);
30 Ball et al., J. Pept. Sci, 1:288-294 (1995); Funakoshi et al, PNAS USA, 88:6981-6985 (1991);

1 Funakoshi et al., J. Chromatog. 638:21-27 (1995); Garcia-Echeverria et al., J. Chem. Soc., Chem.
2 Commun., 779-780 (1995). A preferred cleavable handle is Boc-HN-CH₂-CH₂-SO₂-CH₂-CH₂-
3 O-CO-ONp (Boc-HNCH₂-MSC-) or a functionalized cleavable handle, X-
4 aminoethylsulfonylethylloxycarbonyl (wherein X=CH₃COCH₂CH₂CH₂CONHCH₂-MSC- or
5 X= AOA-NHCH₂-MSC-). (AOA = aminooxyacetal). Another preferred cleavable handle is a
6 CAM ester. See Ceccato, M.L. et al., Tetrahedron Lett. 31:6189-6192 (1990).

7 Cleavable Linker: A cleavable moiety that is capable of being selectively cleaved to
8 monitor the solid phase sequential ligation using mass spectrometry of small samples of the
9 reaction mixture at any point during the ligation procedure, i.e. after ligating of the second
10 peptide segment, after ligating of the third peptide segment, and so forth. The cleavable linker
11 must be stable under coupling and ligating conditions, deprotecting conditions (if needed), and
12 washing conditions. Preferred cleavable linkers include photolabile linkers and TFA-labile
13 linkers.

14 Coupling: Chemoselective reactions involving covalent binding of a first peptide
15 segment to a solid phase.

16 Ligating: Chemoselective reactions involving covalent binding of a peptide segment to a
17 solid phase-bound peptide.

18 Linker: A covalent linkage linking various moieties. For example, a linker may link a
19 first peptide segment and a solid support, and such a linker may optionally comprises any
20 number of moieties, including a cleavable handle, a cleavable linker, complementary functional
21 groups capable of chemoselectively forming a covalent bond (e.g., amino-oxy and ketone to
22 form an oxime).

23 Peptide: A polymer of at least two monomers, wherein the monomers are amino acids,
24 sometimes referred to as amino acid residues, which are joined together via an amide bond. For
25 purposes of this invention, the terms "peptide," "polypeptide," and "protein," are largely
26 interchangeable as all three types can be made via the methods described herein. Peptides are
27 alternatively referred to as polypeptides. Amino acids include the L and D isoforms of chiral
28 amino acids.

29 Peptide segment: A peptide or polypeptide, having either a completely native amide
30 backbone or an unnatural backbone or a mixture thereof, ranging in size from 2 to 1000 amino

1 acid residues, preferably from 2-99 amino acid residues, more preferably from 10-60 amino acid
2 residues, and most preferably from 20-40 amino acid residues. Each peptide segment can
3 comprise native amide bonds or any of the known unnatural peptide backbones or a mixture
4 thereof. Each peptide segment can be prepared by any known synthetic methods, including
5 solution synthesis, stepwise solid phase synthesis, segment condensation, and convergent
6 condensation. The final peptide segment to be added to form the assembled peptide product can
7 be recombinantly expressed.

8 Protecting Group: A chemical moiety capable of protecting a functional group from
9 reacting with another functional group, and removable without damage to the formed amino acid
10 or peptide.

11 Sequential ligation: ligating three or more peptide segments together in order from C-
12 terminus to N-terminus or from the N-terminus to C-terminus, depending on the directionality
13 chosen, to obtain an assembled peptide product. The directionality of the sequential ligations
14 will always start from the solid phase-bound first peptide segment to the last peptide segment to
15 be added to form the assembled peptide product.

16 Solid Phase: A material having a surface and which is substantially insoluble when
17 exposed to organic or aqueous solutions used for coupling, deprotecting, and cleavage reactions.
18 Examples of solid phase materials include glass, polymers and resins, including polyacrylamide,
19 PEG, polystyrene PEG-A, PEG-polystyrene, macroporous, POROS™, cellulose, reconstituted
20 cellulose (e.g. Perloza), nitrocellulose, nylon membranes, controlled-pore glass beads,
21 acrylamide gels, polystyrene, activated dextran, agarose, polyethylene, functionalized plastics,
22 glass, silicon, aluminum, steel, iron, copper, nickel and gold. Such materials may be in the form
23 of a plate, sheet, petri dish, beads, pellets, disks, or other convenient forms. Sheets of cellulose
24 can be used as a solid phase in the present invention to accomplish spot ligation in a spatially
25 addressable array. Many of the examples and embodiments described herein refer to resins,
26 which are a type of solid phase, and one of ordinary skill in the art would understand that such
27 examples are not meant to be limited to resins, but to solid phases in general. The terms solid
28 phase and solid support are used herein interchangeably.

29 Solid Phase-bound Peptide: a solid phase-bound peptide comprises at least one peptide
30 segment bound to a solid phase via any variety of cleavable linkers, handles or moieties. A solid

1 phase-bound peptide can include any of the intermediate peptide products of the sequential
2 ligation reactions, including the final solid-phase bound peptide produced after the final peptide
3 segment is ligated to the penultimate solid phase-bound peptide.

4 Thioacid: An ionizable thioacid moiety, represented by either -COSH or -COS⁺, often
5 referring to a peptide thioacid, represented by "peptide α -COSH" or
6 "peptide α -COS⁺."

7 Thioester: A moiety represented by -COSR, often connected to a peptide. For example,
8 a peptide thioester may be represented as "peptide α -COSR". The R group may be any number
9 of groups, including 1-15 C functionalized alkyl, straight or branched, 1-15 C aromatic
10 structures, 1-4 amino acids or derivatives thereof, preferably wherein the R group is selected
11 such that the peptide- α -COSR is an activated thioester. In a preferred embodiment, R = -
12 CH₃-Ø, -Ø. The term "thioester" is commonly used, but the true IUPAC term is "thioloester."
13 See Matthys J. Janssen, *supra*

14

15 I. SOLID PHASE SEQUENTIAL NATIVE LIGATION OF UNPROTECTED PEPTIDE SEGMENTS IN 16 THE N- TO C-TERMINAL DIRECTION

17 There have been few reports of proteins synthesized by sequential, multiple ligations of
18 three or more unprotected peptide segments. Such sequential ligations of free peptide segments
19 in solution consequently require a purification (e.g. HPLC) after each ligation and typically
20 require temporary protection of one of the functionalities of the middle segments.

21 One aspect of the present invention is a solid phase sequential ligation technique which
22 avoids the need for multiple purifications and the need to temporarily protect the middle peptide
23 segments. This strategy employs (1) the modification of the N-terminal peptide segment with a
24 cleavable handle functionalized with a group capable of chemoselective reaction with the solid
25 support and (2) sequential native chemical ligations of unprotected peptide segments in an N- to
26 C-terminal direction. Native chemical ligation involves reaction of an unprotected peptide
27 segment bearing a C-terminal α -thioester with a second unprotected peptide segment containing
28 an N-terminal Cysteine residue. Thiol exchange yields a thioester-linked intermediate which
29 spontaneously rearranges to a native amide bond at the ligation site. We have determined that a
30 peptide segment bearing an N-terminal Cysteine and a C-terminal thioacid is sufficiently stable

1 under native ligation conditions that it requires no temporary protection of the C-terminal
2 thioacid functionality. Accordingly, these peptide segments can be used as the middle segments
3 in a sequential ligation scheme involving three or more peptide segments as shown in FIG. 1.
4 Once such a middle segment has ligated to the solid phase-bound thioester-containing peptide to
5 generate a solid phase-bound peptide thioacid, the thioacid is easily converted to a thioester and
6 can be reacted with the N-terminal Cysteine of the next peptide segment to be ligated.
7 Alternatively, the incoming peptide segment may have an internal amino acid with a nonnatural
8 side chain bearing amino and thiol moieties on adjacent α atoms, i.e. in a 1,2 relation to one
9 another, and an unreactive, unprotected non-cysteine amino acid residue at its N-terminus, which
10 would lead to a nonlinear assembled peptide. Multiple ligations of distinct peptide segments to
11 form an assembled peptide bound to the solid phase are contemplated. Once all ligations are
12 complete, the linker binding the solid phase-bound peptide to the solid phase is cleaved,
13 releasing the assembled peptide, i.e., the full length peptide. This technique is applied to the
14 total chemical synthesis of a random peptide of artificial sequence (Table 1 in Examples
15 Section), and human Macrophage Migration Inhibitory Factor (MIF), a 115 amino acid cytokine
16 involved in immune system function. See FIG. 16-20.

17

18 A. Peptide Synthesis

19 Peptide segments were synthesized in stepwise fashion by established machine-assisted
20 solid-phase methods on polystyrene resins using *in situ* neutralization/HBTU activation protocols
21 for Boc chemistry (L. Canne et al., Tetrahedron Lett. 38:3361-3364 (1997)) on Boc-aminoacyl-
22 OCH₂-PAM resins, thioester-generating resins (Hojo, et al., Bull. Chem. Soc. Jpn. 64:111-117
23 (1991)), or thioacid-generating resins. After chain assembly was complete, peptides were
24 deprotected and simultaneously cleaved from the resin by treatment with anhydrous HF
25 containing 5% *p*-cresol, lyophilized, and purified by preparative HPLC. The N-terminal peptide
26 segment was modified prior to HF cleavage as outlined in FIG. 17A.

27

28 B. Preparation of the Solid Phase

29 The solid phase is prepared as depicted in FIG. 5A and 5B. FIG. 5A is a scheme for
30 preparing PEGA resin as a solid support. FIG. 5B is a generalized diagram for the preparation

1 of any solid phase. An amino-Spherilose™ (Isco) affinity resin was derivatized with Boc-
2 aminooxyacetic acid as shown in FIG. 17B.

3 Other resins to be used as the solid phase include EAH Sepharose (Pharmacia), Amino
4 PEGA (Novabiochem), CLEAR base resin (Peptides International), long chain alkylamine
5 controlled pore glass (Sigma), HCl•PEG polystyrene (PerSeptive Biosystems), Lysine Hyper D
6 resin (Biosepra), ArgoGel Base resin (Argonaut Technologies). These resins are available in
7 amino-derivatized form or are readily converted to amino-derivatized form.

10 C. Coupling of Modified N-terminal Peptide Segment to Solid Phase.

11 The modified peptide, containing a ketone moiety, as depicted in FIG. 17A, is dissolved
12 in 6M guanidine• HCl, 0.1M Na acetate, 0.15M methionine, pH 4.6 (1.6mM) and added to the
13 aminooxy functionalized solid support, which had previously been thoroughly washed in the
14 same buffer, and allowed to react at room temperature overnight (FIG. 16A, Step #1).

16 II. LIGATION IN THE N- TO C-TERMINAL DIRECTION

17 A. Ligation Reactions. The peptide segment to be ligated to the resin-bound peptide
18 thioester was dissolved in 6M guanidine• HCl, 0.1M Na acetate, 0.15M methionine,
19 0.5% thiophenol, pH 7.5 (3.7-4.0mM) and added to the resin bound peptide thioester, which was

20 thoroughly washed in the same buffer, and allowed to react at room temperature overnight (FIG.
21 16A and 16B, Steps #2 and 4). Preferably the concentration of the first peptide segment can
22 range from 1 to 150 mM; more preferably from 5-100 mM, most preferably from 10-50 mM,
23 depending on the particular peptide segment.

24 One of skill in the art will understand that concentrations of the first peptide segment and
25 the second and other incoming peptide segments can be optimized using routine
26 experimentation. Concentrations of the second and additional incoming peptide segments can
27 range from 1-200 mM, more preferably from 5-100 mM, and most preferably from 10-59 mM,
28 depending on the particular peptide segment.

1 Excess first peptide segment and/or excess incoming peptide segments can be readily
2 removed from the solid phase bound peptide by filtration and washing.

3

4 **B. Conversion of Thioacid to Thioester using Bromoacetic Acid or Iodoacetic Acid.**

5 The use of Bromoacetic acid or Iodoacetic acid is an improved method of generating
6 peptide- α COSR thioesters from peptide- α COSH thioacids. In order to insure solubility of long
7 unprotected peptides, 6 M guanidine-HCL at near pH 4 is used. Reactions is carried out near pH
8 4. Under such conditions, the only group reactive with Bromoacetic acid or Iodoacetic acid is
9 the thioacid. Benzyl bromide, a hydrophobic compound, does not dissolve completely in
10 solution, resulting in slow and heterogeneous reactions. The advantages of using bromoacetic
11 acid or iodoacetic acid are that both are readily soluble in 6 M guanidine-HCL (an aqueous
12 solution) at near pH 4, both result in quick completion of the desired reaction, both elute in the
13 void volume of typical reverse-phase HPLC, and allow processing of large amounts of peptide
14 segments.

15 The resin-bound peptide thioacid is thoroughly washed in 6M guanidine• HCl, 0.1M Na
16 acetate, 0.15M methionine, pH 4.6 and treated with a 50mM solution of bromoacetic acid in the
17 same buffer for 15 min, followed by thorough washing with the pH 4.6 buffer (FIG. 16B, Step
18 #3).

19

20 **C. Cleavage from the Solid Phase.**

21 Cleavable handles useful in the ligations in the N- to C-terminal direction must be capable
22 of being stable to ligation conditions, stable to stepwise solid phase chemistries, able to be
23 covalently linked in unprotected form to the solid phase, and be cleavable without damaging the
24 assembled polypeptide. Any cleavable handles satisfying these requirements can be used,
25 including, but not limited to: MSC handle, photolabile linkers, CAM esters (-OCHCONH-), (-
26 O-CH₂- \emptyset -SO-CH₂-CO-), (-O-CRH-CO- \emptyset -O-CH₂-CO-). For example, (-O-CH₂- \emptyset -SO-
27 CH₂-CO-) may be used as a handle cleavable under any of the following conditions: (1) HF,
28 DMS; (2) SciCl₄, TFA; or red of Sulfoxide and TFA cleavage; (3) NaOH, water; or (4) red of

1 sulfoxide and TBAF in DMF. See Samamen, J. M., J. Org. Chem. 53:561 (1988). As another
2 example, the (-O-CRH-CO-O-CH₂-CO-) may be used as a cleavable handle under any of the
3 following conditions: (1) NaOH, water (CAM Linker); (2) ZnCH₃COOH/Water; (3) photolysis.
4 See Tjoeng et al., Synthesis 897 (1981); Sheehan et al., J. Org. Chem. 38:3771 (1973);
5 Serebryakov et al., Tetrahedron 34:345 (1978); Hendrickson et al., Tetrahedron Lett. 343 (1970);
6 Ceccato, M.L. et al., Tetrahedron Lett. 31:6189-6192 (1990); J. Martinez et al., Tetrahedron Lett.
7 41:739 (1985). One of skill in the art will readily appreciate the suitability of known cleavable
8 handles for the purposes described herein.

9 The following conditions can be used for cleavage of the linker to release the assembled
10 polypeptide from the solid phase, particularly when an MSC handle is used. Aliquots of resin-
11 bound peptide are treated with 6M guanidine• HCl, 0.1M Na acetate, 0.15M methionine,
12 containing 200 mM hydrazine, at pH ~14 for 2 min, followed by washing with an equal amount
13 of 6M guanidine• HCl, 0.1M Na acetate, 0.15M methionine, pH ~2 and an equal amount of 6M
14 guanidine• HCl, 0.1M Na acetate, 0.15M methionine, pH 4.6. The combined eluants of free
15 peptide are analyzed by analytical HPLC and electrospray mass spectrometry (FIG. 16B, Step
16 #5).

17 18 II. SOLID PHASE LIGATIONS IN THE C- TO N-TERMINAL DIRECTION.

19 The discussion regarding N- to C-terminal ligations above applies equally well to C- to
20 N-terminal ligations, except, as shown in FIG. 23, that: (1) the first peptide segment is bound to
21 the solid phase via its C-terminus, i.e. the C-terminal peptide segment of the resulting assembled
22 polypeptide is the one modified with a cleavable handle and (2) the incoming (i.e. second, third,
23 additional) peptide segments do require temporary protection of their N-terminal Cysteine (see
24 steps 2-4). Optionally, all Cysteine residues of the incoming or middle peptide segments can be
25 temporarily protected along with the N-terminal Cysteine.

1 As outlined in the scheme (FIG. 23), the C-terminal peptide segment bearing a cleavable
2 handle is coupled to the solid support by reaction with a corresponding functional group on the
3 solid support (e.g. resin), for example, through an oxime linkage (aminooxyacetyl group on the
4 resin and a ketone [via levulinic acid] on the peptide), or the reverse (aminooxyacetyl group on
5 the peptide and a ketone on the solid phase).

6 Once the first peptide segment is bound to the solid phase as shown in step 1 of FIG. 21,
7 the incoming (second) peptide segment, comprising an N-terminal protected Cys (PG-Cys) and
8 a C-terminal thioester, reacts with the N-terminal unprotected Cys of the resin-bound first
9 peptide segment through the native chemical ligation reaction. After ligation is complete, the
10 protecting group of the N-terminal Cys is removed (step 3 of FIG. 21), and the next peptide
11 segment is added (step 4/2 of FIG. 21). Once all ligations are complete (step 5 of FIG. 21), the
12 handle attaching the sequentially ligated peptide to the resin is cleaved, releasing the full length
13 peptide. This C- to N-terminal technique is applied to the total chemical synthesis of a random
14 peptide of artificial sequence and to human secretory phospholipase A2, group 5 ("PLA2G5"), a
15 118 amino acid enzyme, as described below.

16

17 A. Peptide Synthesis

18 Peptide synthesis for solid phase sequential native chemical ligation in the C- to N-
19 terminal direction is essentially the same as described above for solid phase sequential native
20 chemical ligation in the N- to C-terminal direction.

21 See Example 7 below for details re stepwise solid phase peptide synthesis of the peptide
22 segments.

23

1 **B. Preparation of the Solid Phase**

2 Preparation of the solid phase for the C-to N-terminal direction is identical to that
3 described for the N- to C-terminal direction.

4

5 **C. Coupling of the Modified C-Terminal Peptide Segment to Solid Phase**

6 Conditions for coupling the modified C-terminal peptide segment to the solid support can
7 be identical to that outlined for coupling of the modified N-terminal peptide in the N- to C-
8 terminal ligations as described above.

9

10 **D. Ligation in the C- to N-terminal Direction**

11 Conditions for the native chemical ligation reactions in the C- to N-terminal direction can
12 be identical to that outlined for N- to C-terminal ligations as described above, except that the N-
13 terminal cysteine containing peptide segment is solid phase bound and the incoming thioester
14 containing peptide segment is in solution.

15

16 **E. Cysteine Protecting Groups and Removal**

17 Any of the known protecting groups suitable for protecting the N-terminal Cys of a
18 peptide segment can be used, provided that they are stable to ligation conditions, stable to
19 conditions for adding the linker, and removable from the peptide segment under conditions that
20 are not harmful to the solid-phase bound peptide, the linker, the resin, or the cleavable handle, if
21 used. The protecting groups must also be stable to stepwise solid phase peptide synthesis
22 conditions. An example of a protecting group is ACM (Acetamidomethyl), which provides
23 cysteine side chain protection ($-\text{SCH}_2\text{NHCOCH}_3$), and can be cleaved with mercury(II)acetate,

1 or other suitable reagents. Fmoc (9-Fluorenylmethylcarbamate) provides alpha amino protection,
2 can be cleaved in 20% piperidine in DMF and works well with hydrophilic peptides. DNPE (2-
3 (2,4-dinitrophenyl)ethyl) provides cysteine side chain protection and cleaves in 50% piperidine in
4 DMF. Para-nitrobenzenesulfonyl provides alpha-amino protection, and is cleaved in 1 M DBU/1
5 M beta-mercaptoethanol in DMF. Additional cysteine protecting groups include, but are not
6 limited to, Sulfmoc, NSC, Dde, Boc-Cys(Acm)-OH, Fmoc-Cys-(Mob)-OH, Boc-Cys(Fm)-OH,
7 and Boc-Cys(DNPE)-OH, wherein Acm=acetamidomethyl, Mob = methoxybenzyl, Dnpe = 2-
8 (2,4-dinitrophenyl)ethyl, Fm = 9-fluorenylmethyl. See Protective Groups in Organic Synthesis,
9 Green, T.W. and Wuts, P.G.M. eds, (2d Ed. 1991), particularly p. 293-294, 318-319; R.
10 Merrifield, J. Org. Chem. 43:4808-4816 (1978); V.V. Samukov et al., Tetrahedron Lett.
11 35:7821-7824 (1994); B.W. Bycroft et al., J. Chem. Soc. Chem. Comm. 776-777 (1993); M.
12 Royo et al., Tetrahedron Lett., 33:2391-2394 (1992); S.C. Miller, J. Am. Chem. Soc. 119:2301-
13 2302 (1997). Certain protecting groups can make peptide segments insoluble. For example,
14 certain hydrophobic peptide segments may become insoluble upon addition of a protecting
15 group. One of ordinary skill in the art can readily ascertain the suitability of any particular
16 protecting group for a peptide segment.

17

18 **Removal of Fmoc as a Cys Protecting Group.** One embodiment involves removal of
19 an Fmoc protecting group from the N-terminal Cys of a solid-phase bound peptide. After
20 ligation with a peptide with an N-terminal Fmoc-Cys, the resin bound peptide is washed with 6
21 M guanidine•HCl, 0.1 M NaPi, 0.15 M methionine, pH 7, followed by water, followed by DMF.
22 The resin is then treated with two aliquots of 20% piperidine in DMF, 5 minutes each. The resin

1 is then washed thoroughly with DMF, followed by water, followed by 6 M guanidine•HCl, 0.1
2 M NaPi, 0.15 M methionine, pH 7.

3 **Removal of ACM as a Cys Protecting Group.** After ligation with a peptide with an N-
4 terminal Cys(ACM), the resin bound peptide is washed with 6 M guanidine•HCl, 0.1 M NaPi,
5 0.15 M methionine, pH 7, followed by 3% aqueous acetic acid. The resin is then treated with a
6 solution of mercury(II)acetate in 3% aqueous acetic acid (15mgs/ml) for 30 minutes, followed by
7 washing with 3% aqueous acetic acid. The resin is then washed with 6 M guanidine•HCl, 0.1 M
8 NaPi, 0.15 M methionine, pH 7, followed by treatment with 20% beta-mercaptoethanol in 6 M
9 guanidine•HCl, 0.1 M NaPi, 0.15 M methionine, pH 7 for 30 min. The resin is then washed with
10 6 M guanidine•HCl, 0.1 M NaPi, 0.15 M methionine, pH 7.

12 **F. Cleavage from the Solid Phase**

13 Cleavable handles are used to cleave the solid-phase bound peptide from the solid phase
14 for ligations in the N- to C-terminal direction, in the C- to N-terminal direction, and in the
15 bidirectional approach (both N- to C-terminal ligation and C- to N-terminal ligation). For solid
16 phase sequential native chemical ligations in the C- to N-terminal direction (and for bidirectional
17 ligations using C- to N-terminal ligation), the requirements of cleavable handle are the same as
18 for those useful in the N- to C-terminal direction, with the additional requirement that the
19 cleavable handle be stable under conditions used for removal of the protecting group from the N-
20 terminal cysteine of the solid-phase bound peptide.

21 **Cleavage of a peptide-CAM ester linkage to the solid phase.** Aliquots of resin-bound
22 peptide are washed with 8M urea, 0.1M NaPi, pH 7, followed by treatment for 2 minutes with
23 0.25N NaOH in the same 8M urea buffer (resulting pH~14). The resin is then washed with an

1 equal amount of 0.25N HCl in the same 8M urea buffer (resulting pH~2), followed by thorough
2 washing with the 8M urea buffer. The combined eluants of free peptide are analyzed by HPLC
3 and electrospray mass spectrometry.

4

5 **III. BIDIRECTIONAL SOLID PHASE SEQUENTIAL NATIVE CHEMICAL LIGATION.**

6 Yet another embodiment of the invention relates to bidirectional solid phase protein
7 synthesis that incorporates aspects of both the N- to C-terminus and C- to N-terminus sequential
8 solid phase protein synthesis approaches. In the bidirectional approach, a peptide segment
9 having either or both an N-terminal Cysteine and/or a C-terminal thioester is attached to a solid
10 phase via a side chain of one of its amino acid residues. See FIG. 25A, B, C. The peptide
11 segment can then be ligated at either terminus to a second peptide segment, followed by ligation
12 at the other terminus to a third peptide segment. In this bidirectional approach, if the peptide
13 segment attached to the solid phase has both a protected N-terminal Cysteine and a C-terminal
14 thioester, second and third peptide segments can be added at both ends in subsequent ligations.
15 Additional peptide segments can then be added at either end of the ligated, solid phase bound
16 peptide. The ligations in either direction are accomplished using the methods described herein
17 for ligations in either the C- to N-terminal direction or the N- to C-terminal direction.

18 Alternatively, the first peptide segment attached via one of its internal amino acid
19 residues to the solid phase can be used for only uni-directional ligations. For example, the
20 peptide segment attached to the solid phase can be ligated to a second peptide segment at one
21 terminus, followed by one or more ligations to additional peptide segments at the same terminus
22 of the second peptide segment. In this embodiment, the peptide segment bound to the solid
23 phase can be used for either sequential solid phase native chemical ligations in the C- to N-

1 terminal direction or for sequential solid phase native chemical ligations in the N- to C-terminal
2 direction. In this embodiment, the peptide segment bound to the solid phase can be
3 bidirectionally capable (i.e. having both a protected N-terminal Cysteine and a C-terminal
4 thioester) while being used for unidirectional sequential ligations (i.e. having either a protected
5 N-terminal Cysteine or a C-terminal thioester).

6 The first peptide segment is bound to the solid phase via a side chain of one of its amino
7 acid residues, which is bound to a cleavable handle, which is bound to the solid phase via a
8 functional chemical moiety that is capable of chemoselectively forming a covalent bond with a
9 complementary functional chemical moiety on the solid phase, as illustrated in FIG. 25.

10 For example, the first peptide segment can be bound to the solid phase via the side chains
11 of a lysine, aspartic acid or glutamic acid, in which case a cleavable handle based on
12 functionalities, such as allyloxycarbonyl (alloc) or Fmoc, i.e. cleavable under orthogonal
13 conditions, may be used to connect the peptide segment to the solid phase via the side chain of
14 its lysine, aspartic acid or glutamic acid. As another example, an oxime bond may be formed by
15 the first peptide segment and the solid phase, wherein the first peptide segment comprises either
16 an amino-oxy or ketone chemoselective functional group and the solid phase comprises a
17 complementary chemoselective functional group, such as a ketone or amino-oxy, respectively.
18

19 IV. Use of Cleavable Linkers and Mass Spectrometry to Monitor Ligation Reactions

20 Various known cleavable linkers can be used to monitor the solid phase sequential
21 ligations. These cleavable linkers are placed between the solid phase and the first peptide
22 segment which is covalently bound to the cleavable handle, e.g. solid phase — cleavable linker—
23 cleavable handle—peptide segment. The cleavable linkers are capable of being readily cleaved
24 to permit mass spectrometric analysis of a small portion of solid phase-bound peptide to monitor
25 the coupling and ligation reactions.

26 For example, when the solid phase consists of resin beads, one can take a few resin beads
27 from the reaction mixture after the coupling reaction or after each ligation reaction to determine
28 the extent of reaction. Particularly preferred cleavable linkers include photolabile cleavable
29 linkers for MALDI mass spectrometry, including 3-nitro-4(methylamino)benzoyl-. See FIG.

1 5A. A small aliquot of the reaction mixture is removed for MALDI MS analysis and dried on a
2 slide in mixture with a matrix solution. The laser of the MALDI mass spectrometer cleaves the
3 photolabile linker on the mass spectrometer's stage, permitting mass analysis of the released
4 peptides.

5 Another preferred cleavable linker is one that is cleavable by TFA (trifluoroacetic acid),
6 which is useful for electrospray ionization mass spectrometry. With TFA-cleavable linkers, the
7 peptides are cleaved from the solid phase prior to ESI MS.

8

9

EXAMPLES

Example 1: Preparation of the Solid Phase for N- to C-Terminal Ligations

The preparation of the solid phase is schematically diagrammed in FIG. 5. The solid phase is a resin, for example, Amino PEGA (0.2-0.4 mmol/g swelled in methanol) or an amino-Spherilose affinity resin (15-20 Tmol/ml, 0.6-0.9 mmol/g dry resin), available from Pharmacia, NovaSyn or Isco. The resin (PEGA or Isco) is washed with DMF (dimethylformamide), then is washed briefly with 10% DIEA (diisopropyl ethylamine). Two 30 sec DMF flow washes are used. A photocleavable linker (PCL) (See FIG. 5A) is activated with one equivalent of HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate) and DIEA in DMF for 5-10 min). This activated photocleavable linker is then added to the resin and is left standing at room temperature for ~3 hrs (ninhydrin can be used with Isco).

Two 30 sec. DMF flow washes are used, followed by TFA (1 min x 2), and two more 30 sec. DMF flow washes. The remaining steps are in abbreviated form:

- 10% DIEA (1 min x 2)
- DMF flow wash (30 sec x 2)
- addition of activated Boc-aminoxyacetic acid (activated with one equivalent DIC and N-hydroxysuccinimide in DMF for 30-60 min)
- left standing at room temperature for ~1 hr (ninhydrin can be used with Isco)
- DMF flow wash (30 sec x 2) [resin can be stored at this stage]
- TFA (1 min x 30)
- DMF flow wash (30 sec x 2)
- 10% DIEA (1 in x 2)
- DMF flow wash (30 sec x 2)
- thorough washing with aqueous buffer (6 M GuHCl, 0.1 M Na Acetate, pH 4.6) (1 ml x 5)

Example 2: Preparation of the First Unprotected Peptide Segment for N- to C-terminal Ligations

The following procedures are used to prepare the first peptide segment (N-terminus), which is diagrammed in FIG. 6, 7A and 7B.

- 1 The peptide-resin is swelled in DMF
- 2 - TFA (1 min x 2)
- 3 - DMF flow wash (30 sec x 2)
- 4 - 10% DIEA (1 min x 2)
- 5 - DMF flow wash (30 sec x 2)
- 6 - Addition of MSC handle in DMF
- 7 - leave standing at room temperature for 1 hr
- 8 - add DIEA and leave standing for another hr
- 9 - use ninhydrin test to verify adequate coupling
- 10 - DMF flow wash (30 sec x 2)
- 11 - TFA (1 min x 2)
- 12 - DMF flow wash (30 sec x 2)
- 13 - 10% DIEA (1 min x 2)
- 14 - DMF flow wash (30 sec x 2)
- 15 - addition of activated levulinic acid (activated as the symmetric anhydride with 0.5
- 16 equivalents of DIC in DCM for 5-10 min)
- 17 - leave standing at room temperature for 30 min
- 18 - ninhydrin test to verify adequate ligating
- 19 - DMF flow wash (30 sec x 2)
- 20 - thorough washing with DCM
- 21 - dry on lyophilizer
- 22 - HF cleavage at 0° C for 1 hr using *p*-cresol as a scavenger
- 23 - trituration and washing with cold ethyl acetate
- 24 - dissolve in 50% B and lyophilize
- 25 - purify by preparative HPLC

Solid Phase Sequential Ligations: N- to C-Terminal

3-Random Peptide Segment Model System

Lev-MSCLTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG-COS (1)

+ Resin-*PCL-ONH₂*

↓ 1. pH 4.6, 6M GuHCl, 0.1 M acetate

Resin-*PCL-oxime-MSCLTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG-COS* (1)

↓ 2. pH 4.6, 6M GuHCl, 0.1 M acetate, 50 mM BrAcOH

Resin-*PCL-oxime-MSCLTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG-COSAc* (1)

+ *H-CGFRVREFGDNTA-COS* (2)

↓ 3. pH 7.5, 6M GuHCl, 0.1M phosphate, 0.5% thiophenol

Resin-*PCL-oxime-MSCLTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG*

CGFRVREF-GDNTA-COS (1+2)

↓ 4. pH 4.6, 6M GuHCl, 0.1M acetate, 50mM BrAcOH

Resin-*PCL-oxime-MSCLTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG*

CGFRVREF-GDNTA-COSAc (1+2)

+ *H-CADPSEEWWQKYVSDLELSA-OH* (3)

↓ 5. pH 7.5, 6M GuHCl, 0.1M phosphate, 0.5% thiophenol

Resin-*PCL-oxime-MSCLTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHG*

CGFRVREF-GDNTACADPSEEWWQKYVSDLELSA-OH (1+2+3)

↓ 6. pH 14, 6M GuHCl, 0.1M phosphate, 200mM hydrazine

H-LTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHGCCGFRVREF-
GDNTACADPSEEWWQKYVSDLELSA-OH (1+2+3)

PCL= photocleavable linker

1 **Example 3: Solid Phase Native Chemical Ligation of Random Peptide Segments in**
2 **Aqueous Solution in the N- to C-terminus direction.**

3 The following procedures are used for solid phase ligations in the N- to C-terminus
4 direction, as diagrammed in Table 1. General principals of native chemical ligation are
5 described in WO 96/34878, PCT/US95/05668, incorporated herein by reference.

6 The resin is washed with 6 M guanidine•HCl, 0.1 M Na Acetate, pH 4.6 (1 ml x 5) and
7 drained. The modified N-terminal peptide segment is dissolved in 6 M guanidine•HCl, 0.1 M
8 Na Acetate, pH 4.6 and added to resin and is left standing at room temperature overnight. (The
9 concentration of the first segment is at least 5 mM). The next morning, resin is washed with 6 M
10 guanidine•HCl, 0.1 M Na Acetate, pH 4.6 (1 ml x 5) and drained. A sample of resin is removed
11 for MALDI MS analysis and is washed with 50%B, MeOH, DCM and dried. A sample of resin
12 is removed for base cleavage and is treated with 200 μ l 6 M guanidine•HCl, 0.1 M Na Pi,
13 200mM hydrazine, pH ~14 for 2 min and drained, resin is washed with 200 μ l 6 M
14 guanidine•HCl, 0.1 M Na acetate, 200mM hydrazine, pH ~2 and with 200 μ l 6 M
15 guanidine•HCl, 0.1 M Na Acetate, pH 4.6 and the combined eluants treated with TCEP prior to
16 injection on HPLC.

17 In preparation for addition of the next peptide segment, the resin is washed with 6 M
18 guanidine•HCl, 0.1 M Na Pi, pH 7.5 (1 ml x 5) and drained. The second peptide segment (Cys--
19 COSH) is dissolved in 6 M guanidine•HCl, 0.1 M Na Pi, pH 7.5, 0.5% thiophenol and added to
20 resin. This mixture is left standing at room temperature overnight. The next morning, the resin
21 is washed with 6 M guanidine•HCl, 0.1 M Na Acetate, pH 4.6 (1 ml x 5) and drained. Samples
22 of resin are removed for Maldi and base cleavage and treated as above

23 The solid phase-bound peptide is then converted from COSH to COSAc by treating the
24 resin with 50 mM BrAcOH in 6 M guanidine•HCl, 0.1 M Na Acetate, pH 4.6 for 15 min.

25 The resin is washed with 6 M guanidine•HCl, 0.1 M Na Acetate, pH 4.6 (1 ml x 5) and
26 drained.

27 In preparation for addition of the next peptide segment, the resin is washed with 6 M
28 guanidine•HCl, 0.1 M Na Pi, pH 7.5 (1 ml x 5) and drained. The final peptide segment is
29 dissolved in 6 M guanidine•HCl, 0.1 M Na Pi, pH 7.5, 0.5% thiophenol and added to resin. This

1 reaction mixture is left standing at room temperature overnight. The next morning, the resin is
2 washed with 6 M guanidine•HCl, 0.1 M Na Acetate, pH 4.6 (1 ml x 5) and drained. A sample of
3 resin are removed for monitoring by MALDI MS analysis.

4 The assembled peptide is removed from the solid phase via base cleavage of the
5 cleavable handle from the remaining resin as outlined above only on a larger scale followed by
6 purification by HPLC or desalting on PD-10 column and lyophilization.

7
8 **Example 4: Solid Phase Native Chemical Ligation of C5a(1-74) (74aa) in the N- to C-**
9 **Terminal Direction.**

10 This example describes solid phase sequential native chemical ligation in the N- to C-
11 terminal direction of C5a, Complement Factor 5A. The sequence of C5a is: (SEQ ID NO. ?)
12 TLQKKIEEIAAKYKJSVVKCCYDGACVNNDTCEQRAARISLGPKCIKAFTECCVVAS
13 QLRANISHKDMQLGR.

14 This peptide is prepared using solid phase sequential native ligation of 3 peptide
15 segments: C5a(1-20), C5a(21-46), and C5a(47-74). The procedures used to synthesize C5a by
16 solid phase ligations are identical to those described in the solid phase sequential native ligation
17 of MIF (See Example 5).

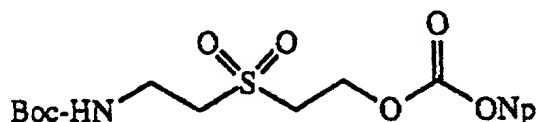
18
19 **Example 5: Solid Phase Sequential Native Chemical Ligation of MIF(1-115) (115 aa) in the**
20 **N-Terminal to C-Terminal Direction.**

21 The sequence of MIF(1-115) is (SEQ.ID.NO.):
22 MPMFIVNTNVPRASVPDGFSEL TQQLAQATGKPPQYIAVHVVPDQLMAFGGSSEPCAL
23 CSLHSIGKIGGAQNRSYSKLLCGLLAERLRISPDRVYINYYDMNAASVGWNNSTFA.

24 This peptide is prepared using solid phase sequential native ligation of 3 peptide segments:
25 MIF(1-59), MIF(60-80) and MIF(81-115). See FIG. 16-20.

1 **Step #1:** The first unprotected peptide segment, MIF(1-59) is coupled to a solid phase as
2 depicted in **FIG. 18**. The coupling conditions are 6M guanidine• HCl, 0.1M NaAcetate, 0.15M
3 Methionine, pH 4.6, 24 hours.

4 The MSC handle used is:



10 This cleavable handle is based on methylsulfonylethoxycarbonyl (MSC) amine protecting
11 group. It is easily added to unprotected amino terminus of peptide-resins, survives HF
12 deprotection and cleavage from the resin, is quickly and cleanly cleaved by aqueous base, and is
13 designed with a protected amine which can be derivatized with a variety of functionalities.

14 **Step #2:** The second unprotected peptide segment (Cys60-MIF(61-80)-COSH) is then
15 ligated to the solid phase-bound first unprotected peptide segment, under the conditions 6 M
16 guanidine• HCl, 0.1M NaPi, 0.5% thiophenol, 0.15M Methionine, pH 7.5, 24 hours.

17 **Step #3:** The solid phase-bound peptide, MIF(1-80)-COSH, is then activated to the
18 thioester under the following conditions: 50 mM BrCH₂COOH, 6M guanidine• HCl, 0.1M
19 NaAcetate, 0.15M Methionine, pH 4.6, 15 min.

20 **Step #4:** The third unprotected peptide segment (Cys81-MIF82-115-COOH) is ligated to
21 the solid phase-bound peptide with 6 M guanidine• HCl, 0.1M NaPi, 0.5% thiophenol, 0.15M
22 Methionine, pH 7.5, 24 hours.

23 **Step #5:** The MIF(1-115) bound to the solid phase is then cleaved from the solid support
24 by base cleavage of the cleavable handle under the cleaving conditions: 6 M guanidine• HCl,
25 0.1M NaAcetate, 0.15M Methionine, 200 mM hydrazine, at pH~14 for 2 min., followed by 6 M
26 guanidine• HCl, 0.1M NaAcetate, 0.15M Methionine, 200 mM hydrazine, at pH~2. The
27 expected mass of the assembled peptide MIF(1-115) released upon base cleavage is 12450 Da.
28 **FIG. 20C and 20D** are mass spectra of the assembled peptide having an expected mass of
29 12450. **FIG 20D** is a reconstruction of the mass spectrum of **FIG 20C**. **FIG 20B** is an HPLC
30 chromatogram of the assembled peptide.

1

2 **Example 6: Solid Phase Native Chemical Ligation of Phospholipase A2, group 5(1-118)**
3 **(118aa) in the C- to N-terminal Direction.**

4 The sequence of Phospholipase A2, group 5 (PLA2G5) is: (SEQ ID NO:):

5 GLLDLKSMIEKVTGKNALTNYGFGCYCGWGGRGTPKDGTDWCCWAHDHCYGRLEE
6 KGCNIRTQSYKYRFAWGVVTCEPGPFCHVNLACDRKLVYCLKRNLRSPNPQYQYFPN
7 ILCS.

8 This peptide is prepared using solid phase sequential native ligation of 4 peptide segments:
9 PLA2G5 (1-25), PLA2G5 (26-58), PLA2G5(59-87) and PLA2G5 (88-118). The procedures
10 used to synthesize PLA2G5 by solid phase ligations are identical to those used for synthesizing
11 the random sequence using ACM protection of the N-terminal Cys residues of the middle
12 segments, as described in **Example 9**. See **FIG. 22** for the reaction scheme. The Cam ester
13 derivative is synthesized and incorporated into the C-terminal peptide segment according to the
14 diagrams in **FIG. 23, 24/FIG. 27**. The assembled polypeptide, PLA2G5 (1-118), was folded
15 and assayed for biological activity. It had the full activity of a recombinantly expressed
16 PLA2G5.

17

18 **Example 7: Preparation of Modified C-terminal Peptide Segment (on-resin CAM linker**
19 **synthesis) (FIG. 27)**

20 The commercial resin of choice (MBHA, any Boc-AA-OCH₂-Pam resin) is swelled in DMF

21 -TFA (1 min x 2) (not necessary if working with MBHA resin)

22 -DMF flow wash (30 sec x 2)

23 -addition of activated Boc-Lys(Fmoc)-OH (HBTU/DIEA activation), check for completion of
24 reaction after 10-15 minutes by ninhydrin test

25 -DMF flow wash (30 sec x 2)

26 -TFA (1 min x 2)

- 1 -DMF flow wash (30 sec x 2)
- 2 -10% DIEA in DMF (1 min x 2)
- 3 -addition of activated bromoacetic acid (activated as the symmetric anhydride with 0.5
- 4 equivalents of DIC in DCM for 5-10 minutes), check for completion of reaction after 30 minutes
- 5 by ninhydrin test
- 6 -DMF flow wash (30 sec x 2)
- 7 -addition of first Boc-protected amino acid of the sequence(Boc-AA-OH) 2M in 20% DIEA in
- 8 DMF. Leave standing at room temperature for 3 hrs.
- 9 -DMF flow wash (30 sec x 2)
- 10 -synthesize rest of the sequence by standard protocols for Boc chemistry
- 11 -remove Fmoc group by treating with 20% piperidine in DMF (5 min x 2)
- 12 -DMF flow wash (30 sec x 2)
- 13 -addition of activated levulinic acid (activated as the symmetric anhydride with 0.5 equivalents
- 14 of DIC in DCM for 5-10 min), check for completion of reaction after 30 minutes by ninhydrin
- 15 test
- 16 -DMF flow wash (30 sec x 2)
- 17 -thorough washing with DCM
- 18 -thoroughly dry resin
- 19 -HF cleavage at 0°C for 1 hr using p-cresol as a scavenger
- 20 -trituration and washing with cold ethyl ether
- 21 -dissolve in aqueous HPLC buffer and lyophilize
- 22 -purify by preparative HPLC

**1 Example 8: Solid Phase Native Chemical Ligation of Random Peptide Segments in the C-
2 to N-terminal Direction using Fmoc protection (See FIG. 28)**

3 The following procedures can be used for solid phase ligations in the C- to N-terminal
4 direction, as diagramed in Table 2. By example, a random peptide of (SEQ ID NO: ?):
5 ALTKYGFYGCYGRLEEKGCADRKNILA can be ligated in three peptide segments (from C-
6 to N-terminal direction): segment 1= CADRKNILA; segment 2 = CYGRLEEKG; and segment
7 3 = ALTKYGFYG.

8 The resin is washed with 6M Gu•HCL, 0.1M Na Acetate, pH 4.6 (1 ml x 5) and drained. The
9 modified C-terminal peptide segment (first peptide segment) is dissolved in 6M Gu•HCL, 0.1M
10 Na Acetate, pH 4.6 (5 mM first peptide segment) and added to the resin and is left standing at
11 room temperature overnight. The resin is washed with 6M Gu•HCL, 0.1M Na Acetate, pH 4.6
12 (1 ml x 5) and drained. A sample is removed for base cleavage and is treated with 8M urea,
13 0.1M NaPi, pH 7, treated for 2 minutes with 0.25N NaOH in the same 8M urea buffer (resulting
14 pH~14), washed with an equal amount of 0.25N HCl in the same 8M urea buffer (resulting
15 pH~2), and the combined eluants treated with TCEP prior to injection on HPLC.

16 In preparation for addition of the next segment, the resin is washed with 6M Gu•HCL, 0.1M
17 NaPi, pH 7.0 (1 ml x 5) and drained. The second peptide segment (Fmoc-Cys-peptide-COSR) is
18 dissolved in 6M Gu•HCL, 0.1M NaPi, pH 7.0, 0.5% thiophenol (to at least 10 mM to 50 mM
19 second peptide segment) and added to the resin. the mixture is left standing at room temperature
20 overnight. The resin is washed with 6M Gu•HCL, 0.1M NaPi, pH 7.0 (1 ml x 5), water (1 ml x
21 5), DMF (1 ml x 5), and the Fmoc protecting group removed by treating with two aliquots of
22 20% piperidine in DMF (5 min each). The resin is then washed with DMF (1 ml x 5), water (1

1 ml x 5), and 6M Gu•HCl, 0.1M NaPi, pH 7.0 (1 ml x 5). A sample of resin is removed and base
2 cleaved as above.

3 The final peptide segment is dissolved in 6M Gu•HCl, 0.1M NaPi, pH 7.0, 0.5% thiophenol
4 and added to the resin. This mixture is left standing at room temperature overnight. The resin is
5 then washed with 6M Gu•HCl, 0.1M NaPi, pH 7.0 and the assembled peptide is removed from
6 the solid phase via base cleavage of the cleavable handle from the remaining resin as outlined
7 above only on a larger scale followed by purification by HPLC or deslating on PD-10 column
8 and lyophilization.

9 These methods can be applied to make any peptides having cysteine residues.

10

11 **Example 8A: Solid Phase Native Chemical Ligation of Random Peptide Segments in the C-**
12 **to N-terminal Direction using DNPE protection**

13 DNPE (2-(2,4-dinitrophenylethyl)) is another cysteine side chain protecting group which
14 can be used for ligations in the C- to N-terminal direction. Example 8 was repeated using DNPE
15 as the protecting group. The conditions for solid phase chemical ligation of random peptide
16 segments in the C- to N-terminal direction were identical to those used for Example 8 above
17 except that in the removal of the DNPE protecting group, 50% piperidine is used.

18

19 **Example 9: Solid Phase Native Chemical Ligation of Random Peptide Segments in the C-**
20 **to N-terminal Direction using ACM protection**

21 The following procedures are used for solid phase ligations in the C- to N-terminal direction,
22 as diagramed in Table 3. The same random polypeptide described in the Example above is
23 ligated.

1 The resin is washed with 6M Gu•HCL, 0.1M Na Acetate, pH 4.6 (1 ml x 5) and drained. The
2 modified C-terminal peptide segment is dissolved in 6M Gu•HCL, 0.1M Na Acetate, pH 4.6 and
3 added to the resin and is left standing at room temperature overnight. The resin is washed with
4 6M Gu•HCL, 0.1M Na Acetate, pH 4.6 (1 ml x 5) and drained. A sample is removed for base
5 cleavage and is treated with 8M urea, 0.1M NaPi, pH 7, treated for 2 minutes with 0.25N NaOH
6 in the same 8M urea buffer (resulting pH~14), washed with an equal amount of 0.25N HCl in the
7 same 8M urea buffer (resulting pH~2), and the combined eluants treated with TCEP prior to
8 injection on HPLC

9 In preparation for addition of the next segment, the resin is washed with 6M Gu•HCl, 0.1M
10 NaPi, pH 7.0 (1 ml x 5) and drained. The second peptide segment (Fmoc-Cys-peptide-COSR) is
11 dissolved in 6M Gu•HCl, 0.1M NaPi, pH 7.0, 0.5% thiophenol (to at least 10 mM second peptide
12 segment) and added to the resin. The mixture is left standing at room temperature overnight.
13 The resin is washed with 6M Gu•HCl, 0.1M NaPi, pH 7.0 (1 ml x 5), 3% acetic acid in water (1
14 ml x 5), and the ACM protecting group removed by treating with mercury(II)acetate in 3% acetic
15 acid in water (15 mgs/ml) for 30 min. The resin is then washed with 3% acetic acid in water (1
16 ml x 5), 6M Gu•HCl, 0.1M NaPi, pH 7.0 (1 ml x 5), and treated with 20% beta-mercaptoethanol
17 in 6M Gu•HCl, 0.1M NaPi, pH 7.0 for 30 min, followed by washing with 6M Gu•HCl, 0.1M
18 NaPi, pH 7.0 (1 ml x 5). A sample of resin is removed and base cleaved as above.

19 The final peptide segment is dissolved in 6M Gu•HCl, 0.1M NaPi, pH 7.0, 0.5% thiophenol
20 and added to the resin. This mixture is left standing at room temperature overnight. The resin is
21 then washed with 6M Gu•HCl, 0.1M NaPi, pH 7.0 and the assembled peptide is removed from
22 the solid phase via base cleavage of the cleavable handle from the remaining resin as outlined

- 1 above only on a larger scale followed by purification by HPLC or deslating on PD-10 column
- 2 and lyophilization.

Table 2

Polymer-Supported Ligations
C- to N- Terminal Direction
Fmoc Protection

H-CADRKNILA-CAM-Lys(*Levulinic acid*)-NH₂ (1)

+ Resin- ONH₂

↓ 1. pH 4.6, 6M Gu•HCl, 0.1 acetate

H-CADRKNILA-CAM-Lys-oxime-Resin (1)

+ *Fmoc*-CYGRLEEKG-COSR (2)

↓ 2. pH 7.5, 6M Gu•HCl, 0.1M phosphate, 0.5% thiophenol

Fmoc-CYGRLEEKGCADRKNILA-CAM-Lys-oxime-Resin (1+2)

↓ 3. 20% piperidine/DMF

H-CYGRLEEKGCADRKNILA-CAM-Lys-oxime-Resin (1+2)

+ *H*-ALTKYGFYG-COSR (3)

↓ 4. pH 7.5, 6M Gu•HCl, 0.1M phosphate, 0.5% thiophenol

H- ALTKYGFYGCYGRLEEKGCADRKNILA-CAM-Lys-oxime-Resin (1+2+3)

↓ 5. pH 14, 8M Urea, 0.1M phosphate, 0.25N NaOH

H- ALTKYGFYGCYGRLEEKGCADRKNILA-OH

Table 3

Polymer-Supported Ligations

C- to N- Terminal Direction

ACM Protection

H-CADRKNILA-CAM-Lys(Levulinic acid)-NH₂ (1)

+ Resin- *ONH₂*

↓ 1. pH 4.6, 6M Gu•HCl, 0.1 acetate

H-CADRKNILA-CAM-Lys-oxime-Resin (1)

+ *H-C(ACM)YGRLEEKG-COSR* (2)

↓ 2. pH 7.5, 6M Gu•HCl, 0.1M phosphate, 0.5% thiophenol

H-C(ACM)YGRLEEKGCADRKNILA-CAM-Lys-oxime-Resin (1+2)

↓ 3. a. mercury(II)acetate in 3% Aq. AcOH
b. 20% mercaptoethanol in pH 7.5, 6M Gu•HCl, 0.1M phosphate

H-CYGRLEEKGCADRKNILA-CAM-Lys-oxime-Resin (1+2)

+ *H-ALTKYGFYG-COSR* (3)

↓ 4. pH 7.5, 6M Gu•HCl, 0.1M phosphate, 0.5% thiophenol

H- ALTKYGFYGCYGRLEEKGCADRKNILA-CAM-Lys-oxime-Resin (1+2+3)

↓ 5. pH 14, 8M Urea, 0.1M phosphate, 0.25N NaOH

H- ALTKYGFYGCYGRLEEKGCADRKNILA-OH

1 Example 10: Bidirectional Solid Phase Sequential Native Chemical Ligation

2 This example illustrates one of the embodiments of the bidirectional solid phase protein
3 ligation approach, namely the situation starting with a first peptide segment bound to the solid
4 phase, wherein the first peptide segment is a "middle piece" of the target protein desired, i.e. the
5 first peptide segment, bound to the solid phase, is used for ligations at both its N-terminal
6 Cysteine and its C-terminal thioester.

7 Starting with one of the middle pieces of the target protein, a cleavable linker is added to
8 the side chain of one of the amino acid residues of the middle piece. The side chain of any
9 amino acid residue having a protectable functional group can be used, including, preferably
10 Aspartic Acid or Glutamic Acid. Most preferably, a Lysine amino acid residue is used. For
11 example, a CAM ester cleavable handle or any other carboxylic acid protecting group may be
12 adapted to attach the first peptide segment to the solid phase through the side chain of Aspartic
13 or Glutamic Acid. One of skill in the art will readily appreciate the necessary chemistries for
14 accomplishing this step.

15 For example, the synthesis of a first peptide segment to be attached to the solid phase
16 via an internal amino acid is illustrated in FIG. 25C. Starting with an appropriate solid phase
17 (thioester or thiacid generating), the first peptide segment is synthesized using standard Boc
18 protocols until the Lysine residue of choice is reached. Using Boc chemistry, a Lysine with its
19 side chain amine protected with an Fmoc group (Boc-Lys(Fmoc)-OH) is inserted at the
20 appropriate location during solid phase stepwise peptide synthesis, followed by continued
21 synthesis to the end of the first peptide segment. The Fmoc protecting group is removed at the
22 end of the stepwise peptide synthesis and the cleavable handle coupled to the side chain amine
23 (step B of FIG. 25C).

1 This method is much the same as the procedure outlined in FIG. 24, with the following
2 differences: the levulinic acid in step 4 is replaced with the cleavable handle and the 20%
3 piperidine used to cleave the Fmoc group (also part of step 4) is replaced with a much smaller
4 concentration of an alternative base such as 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU), e.g. 1-2
5 equivalents of DBU in DMF. The reason is the middle peptide segments, regardless of whether
6 they generate thioacids or thioesters upon cleavage from the resin, are connected to the resin by a
7 thioester which would be cleaved in the presence of 20% piperidine.

8 For this particular strategy, the MSC handle is preferred, although other cleavable
9 handles can be used. Attachment to the side chain amine of a lysine residue and further
10 modification of the linker with an appropriate functional group capable of reacting with a
11 corresponding group on the solid phase ligation resin would be generally as outlined in FIG.
12 17A, with the exception that the amine of the MSC handle should be protected with an Fmoc
13 instead of a Boc group. Since attachment to the peptide segment is through an internal amino
14 acid residue, the N-terminal amino acid would be Boc protected and it is not possible for the N-
15 terminal amino group and the amino group of the MSC cleavable handle to be protected by the
16 same group. Removal of the Fmoc group on the MSC cleavable handle would also need to be
17 done with DBU instead of piperidine. As in FIG. 17A, levulinic acid is preferred for coupling to
18 the linker with a corresponding aminooxyacetyl group on the solid support (FIG. 17B).

19 Two versions of the first peptide segment to be coupled to the resin are described below.

20 **First Version.** The first peptide segment has an unprotected N-terminal cysteine and a
21 C-terminal thioacid (FIG. 25A). The second peptide segment (step 2. in FIG.25A), to be ligated
22 to the first peptide segment, is a peptide with a C-terminal thioester and optionally a protected N-
23 terminal Cysteine (if additional C- to N-terminal ligations are desired), wherein the C-terminal

1 thioester is capable of reacting with the N-terminal Cys of the first peptide segment (i.e. in the C-
2 to N-terminal direction). This step can be multiply repeated with additional peptide segments
3 added in the C- to N-terminal direction, if desired, provided that the internal incoming peptide
4 segments each comprise a protected N-terminal Cysteine, which can be deprotected according to
5 the standard C- to N-terminal solid phase native chemical ligation steps outlined in FIG. 21 (the
6 final peptide segment to be added at the N-terminus of the resulting product need not have an N-
7 terminal Cysteine). After ligation is complete, the C-terminal thioacid of the resulting solid-
8 phase bound peptide (i.e. ligation product of first and second peptide segments) is then converted
9 to a thioester with bromoacetic acid (as outlined in N- to C-terminal ligations in Table 1 and
10 diagrammed as step 3 of FIG 25A). The next step (step 4 of FIG.25A) comprises ligation of the
11 solid-phase bound peptide to a third peptide segment with an N-terminal Cys. This step can
12 optionally be repeated, to add additional incoming peptide segments in the N- to C-terminal
13 direction, if desired, provided that the internal incoming peptide segments each comprise an
14 unprotected N-terminal Cysteine and a C-terminal thioacid, with conversion of the thioacid to
15 thioester after the ligation is complete and prior to addition of the next peptide segment. The
16 final peptide segment to be added at the C-terminus of the resulting product need not have a C-
17 terminal thioacid.

18 One of skill in the art will appreciate that multiple ligations can subsequently be
19 performed in both directions if the appropriate protecting groups and other appropriate
20 chemistries are used on the middle piece or the solid-phase bound peptide. These additional
21 steps are identical to the strategies used for the individual directions, i.e. N-terminal unprotected
22 Cys plus C-terminal thioester for the N- to C- direction and N-terminal Cys(ACM) plus C-
23 terminal thioester for the C- to N-terminal direction. Assuming the MSC linker is used, cleavage

1 of the full length product from the resin would be in basic solution (pH 12-14) as outlined in step
2 6 in Table 1. However, the preferred approach is to complete all ligation steps necessary for one
3 direction, followed by the ligation steps for the other direction. As long as the solid phase bound
4 peptide has either a protected N-terminal Cysteine or a C-terminal thioacid, ligations can proceed
5 in either direction provided that the appropriate strategies as described herein are followed. If
6 the solid phase bound peptide has both an unprotected N-terminal Cysteine and a C-terminal
7 thioester, any attempts at ligating to an additional incoming peptide segment will result in
8 cyclization of the solid-phase bound peptide.

9 **Second Version.** The second version of this scheme involves starting with ligation in
10 the N- to C-terminal direction, followed by ligation in the opposite direction, as shown in FIG.
11 25B. The first peptide segment to be coupled to the resin comprises a temporarily protected N-
12 terminal Cys and a C-terminal thioester. The ligation of a second peptide segment to the first
13 peptide segment is then in the N- to C-terminal direction. Any subsequent ligations in the C- to
14 N-terminal direction would first require removal of the protecting group.

15 Except for the attachment of the first peptide segment to the solid support, this strategy
16 merely combines the procedures for N- to C- and C- to N-terminal ligations (described above).

17

18 References

19 S. Funakoshi et al., Chemoselective one-step purification method for peptides
20 synthesized by the solid-phase technique, Proc. Nat. Acad. Sci. USA, 88:6981-6985 (Aug. 1991).

21 S. Funakoshi et al., Affinity purification method using a reversible biotinylating reagent
22 for peptides synthesized by the solid-phase technique, J. Chromatog. 638:21-27 (1993).

23 M. Mutter et al., Pseudo-prolines (psi Pro) for accessing inaccessible peptides, Pept. Res.
24 8(3):145-153 (1995).

- 1 M. Baca et al., Chemical ligation of cysteine-containing peptides: synthesis of a 22 kDa
2 tethered dimer of HIV-1 protease, *J. Am. Chem. Soc.* 117(7): 1881-1887 (1995).
- 3 J. Camarero et al., Chemical Ligation of Unprotected Peptides Directly From a Solid
4 Support, *J. Peptide Res.* 51: 303-316 (1998).
- 5 L. Canne et al., Total Chemical Synthesis of a Unique Transcription Factor-Related
6 Protein: cMyc-Max, *J. Am. Chem. Soc.* 117:2998-3007 (1995).
- 7 C. Cho et al., An Unnatural Biopolymer, *Science* 261:1303-1305 (1993).
- 8 P. Dawson et al., Synthesis of Proteins by Native Chemical Ligation, *Science* 266:776-
9 779 (1994).
- 10 N. Fotouhi et al., *J. Org. Chem.* 54:2803-2817 (1989).
- 11 G. Barany and R.B. Merrifield, A New Amino Protecting Group Removal by Reduction.
12 Chemistry of the Dithiasuccinoyl (Dts) Function, *J. Am. Chem. Soc.*, 99(22):7363-7365 (1977).
- 13 C. Hennard and J. Tam, Sequential Orthogonal Coupling Strategy for the Synthesis of
14 Biotin Tagged β Defensin, Abstract P118, Fifteenth American Peptide Symposium, June 14-19,
15 1997.
- 16 C. Hyde et al., Some difficult sequences made easy, A study of interchain association in
17 solid-phase peptide synthesis, *Int. J. Peptide Protein Res.* 43:431-440 (1994).
- 18 W. Lu et al., *Biochemistry*, 36(4):673-679 (1997).
- 19 C.-F. Liu and J. Tam, Peptide segment ligation strategy without use of protecting groups,
20 *Proc. Nat. Acad. Sci. USA*, 91: 6584-6588 (1994).
- 21 C.-F. Liu and J. Tam, Chemical ligation approach to form a peptide bond between
22 unprotected peptide segments. Concept and model study, *J. Am. Chem. Soc.* 116(10):4149-4153
23 (1994). Schnolzer et al., *Science* 256:221-225 (1992)
- 24 Rose et al. *J. Am. Chem. Soc.* 116:30-34 (1994)
- 25 Liu et al., *Proc. Natl. Acad. Sci. USA* 91:6584-6588 (1994).
- 26 Dawson et al. *Science* 266:77-779 (1994).
- 27 PCT/US95/05668, WO 96/34878
- 28 Sakakibara S., *Biopolymers (Peptide Science)*, 37:17-28 (1995).
- 29 Tam et al., *PNAS USA*, 92:12485-12489 (1995).

1 T. Muir, A Chemical approach to the construction of multimeric protein assemblies,
2 Structure 3:649-652 (1995).

3 R. Merrifield, Solid Phase Peptide Synthesis: The Synthesis of a Tetrapeptide, J. Am.
4 Chem. Soc., 85:2149-2154 (1963).

5 H. Muramatsu et al., Localization of Heparin-Binding, Neurite Outgrowth and Antigenic
6 Regions in Midkine Molecule, Biochem. And Biophys. Res. Commn. 203 (2):1131-1139 (1994).

7 PCT/US94/07222, WO 95/00846, Published January 5, 1995.

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10 All publications and patent applications mentioned in this specification are herein
11 incorporated by reference to the same extent as if each individual publication or patent
12 application was specifically and individually indicated to be incorporated by reference.

13

14 The invention now being fully described, it will be apparent to one of ordinary
15 skill in the art that many changes and modifications can be made thereto without departing from
16 the spirit or scope of the appended claims.